The prognostic role of the non-canonical NF-kappa B pathway in renal cell carcinoma patients

Running Title – The non-canonical NFκB pathway in renal cancer

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ABSTRACT

Background

8000 cases of renal cancer are diagnosed each year in the UK, with a five-year survival rate of 50%. Treatment options are limited; a potential therapeutic target is the non-canonical NF-kappa B pathway. This pathway plays a role in multiple oncogenic processes in solid tumors. The aim of this study was to investigate the non-canonical NF-kappa B pathway in renal cell carcinoma.

Materials and Methods

NIK, IKKα and RelB were investigated via immunohistochemistry in a cohort of 192 patients with clear cell renal cancer.

Results

High cytoplasmic NIK was associated with poorer cancer-specific survival (p=0.006) and 10-year survival stratified from 85% (low) to 65% (high, p=0.005). Similarly, high cytoplasmic RelB was associated with poorer cancer-specific survival (p=0.041) and 10-year survival stratified from 88% (low) to 73% (high, p=0.030). When clinicopathological characteristics were assessed, cytoplasmic NIK was associated with survival (p=0.014). Whereas, cytoplasmic RelB was associated with increased tumor grade (p=0.020) and decreased inflammation (p=0.019). Upon multivariate analysis cytoplasmic NIK was independently associated with cancer-specific survival (p=0.009).

Conclusions

The non-canonical NF-kappa B pathway is associated with poorer cancer-specific survival in renal cell carcinoma patients, making it a viable target for therapeutic

intervention. Furthermore, cytoplasmic NIK is a potential prognostic biomarker for this disease.

KEY OF DEFINITIONS FOR ABBREVIATIONS

CSS = Cancer specific survival

IRI = Ischemia-reperfusion injury

NFκB = Nuclear factor kappa B

RCC = Renal cell carcinoma

INTRODUCTION

Renal cell carcinoma (RCC) is the 8th most common cancer in the UK with a 5-year survival rate of around 50%.[1] Incidence rates have been rising due to the development of modern imaging techniques for earlier cancer detection as well as a rise in risk factors such as smoking and obesity. Chemotherapy is largely ineffective as RCCs have high expression levels of the multi-drug resistance protein, P-glycoprotein. Commonly used modes of first-line systemic therapy include Sunitinib and Pazopanib (tyrosine kinase inhibitor), Bevacizumab (vascular endothelial growth factor inhibitor), interferon α (immunotherapy), and Temsirolimus (mammalian target of rapamycin inhibitor).[2] The ineffectiveness of chemotherapy limits the number of available treatment options for patients, thus research into pathways contributing to RCC tumorigenesis is necessary to identify drug targets and develop potential therapeutics.

Nuclear factor kappa B (NFκB) is a family of transcription factors which can act via either the canonical or non-canonical pathway. The non-canonical pathway activates NIK, which subsequently phosphorylates IKKα. IKKα then phosphorylates p100, leading to ubiquitination of p100 and release of active p52:RelB dimers. The p52:RelB dimers can then translocate to the nucleus where they drive gene transcription.[3, 4] The functions of genes transcribed by NFκB can be divided into 4 broad categories, immunoregulatory and inflammatory genes, anti-apoptotic genes, genes promoting cell proliferation and negative regulators of NFκB, all of which are important in RCC. It is also known that dysregulation of these target genes lead to tumorigenesis in a variety of cancers.[5]

The importance of the non-canonical pathway has been demonstrated in various types of solid tumors. In breast cancer, the non-canonical pathway can be activated via the RANK ligand. Either inhibiting RANK or constitutive RANK activation causes elevation of NFκB activity, which subsequently stimulates cell proliferation via increased transcription of cyclin D1.[5] In glioblastoma this pathway is implicated to cause a very aggressive subtype. In mouse xenografts, NIK was shown to cause changes in cell conformation, increasing invasive capacity of tumor cells.[6] Studies looking at the role of the non-canonical pathway in renal cells have also been performed, but have not specifically assessed RCC. In renal cells, Tumor Necrosis Factor-like Weak Inducer of Apoptosis (TWEAK) can activate the non-canonical NFκB pathway to induce production of inflammatory mediators, as well as induction of apoptosis during kidney injury and cell proliferation.[7, 8] This pathway has been implicated in various renal diseases such as diabetic nephropathy, glomerular disease and acute kidney injury but its role in RCC is still unclear.[9]

Therefore, this study aimed to investigate the role of the non-canonical NFκB pathway in clear cell RCC, using immunohistochemistry to assess protein expression of NIK, IKKα and RelB. Expression was then correlated with patient survival and clinical outcome measures.

METHODS

Patient cohorts

The cohort consist of 192 patients within the Greater Glasgow National Health Service (NHS) Trust who were diagnosed with clear cell RCC and underwent complete resection of tumor via nephrectomy between 1997 and 2008. Patients were staged and graded according to the TMN classification and Fuhrman grading respectively. Ethical approval for use of renal tissue and patient data was obtained from the NHS Greater Glasgow & Clyde Biorepository and West of Scotland Research Ethics Service.

Immunohistochemistry

Immunohistochemical analysis of NIK, IKKα and RelB were carried out using previously constructed clear cell RCC tissue microarray (TMA).

Sections were dewaxed in Histoclear then rehydrated using graded alcohols. Antigen retrieval was performed using citrate buffer pH6 under pressure for 5 minutes before cooling for 20 minutes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 minutes. 5% horse serum (Vector Laboratories) for IKKα and RelB or 10% Casein for NIK were applied for 30 minutes at room temperature as a blocking solution. Slides were incubated overnight at 4ºC with IKKα (Genway), RelB (Cell Signaling Technology) and NIK (abcam) at concentrations of 1:2000, 1:100 and 1:250, respectively. Envision (Dako) was added to the sections for 30 minutes at room temperature before washing in TBS. DAB substrate was added for five minutes until color developed before washing in running water for ten minutes. Slides were then

counterstained in hematoxylin for 60 seconds and blued with Scotts' tap water before being dehydrated through a series of graded alcohols. Cover slips were applied using distrene, plasticizer, xylene (DPX).

Scoring

Stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualized on Slidepath Digital Image Hub (Leica Biosystems, Milton Keynes, UK). Assessment of NIK, IKKα and RelB expression was performed by a single examiner (J.L) blinded to clinical data at x20 magnification (total magnification x400) using the weighted histoscore.[10] The weighted histoscore is calculated as follows: 0x% not stained + 1x% weakly stained + 2x% moderately stained + 3x% strongly stained. This gives a range of scores from 0 to 300 and is calculated individually for nuclear, membrane and cytoplasmic staining. To ensure reproducibility, 10% of tumors were co-scored by a co-investigator (J.E.).

Statistical analysis

Statistical analysis was carried out using SPSS software. For all statistical analyses, the p-value was set at <0.05. Only patients with a score for IKKα, NIK and RelB were included in the analysis (n=174). The histoscores for NIK and IKKα where dichotomized using the median as the cut-off, whereas RelB was dichotomised using the lower quartile as the cut-off. The relationship between clinicopathological characteristics and protein expression was examined using the chi-square test for linear

trend. The relationship between expression and cancer-specific survival was examined using the Kaplan-Meier method. The log rank test was utilized to compare significant differences between subset groups using univariate analysis. Multivariate cox regression analysis was performed to identify those factors that were independently associated with cancer specific survival (CSS).

RESULTS

The cohort consisted of 192 RCC patients of which, 135 were alive at the last follow-up, 36 died of RCC and 20 died of other causes. Median age of diagnosis is 61 years. The length of follow up ranges from 0.1 to 182 months, with a mean follow up time of 74 months. Mean CSS is 35 months (range 0.1 - 98 months).

For this cohort a tissue-microarray was utilized and expression of NIK, IKKα and RelB was observed in the tumor cell membrane, cytoplasm and nucleus (Figure 1). Only patients with a score for all three proteins were included in the analysis (n=174). Expression was divided into quartiles and the median used as the cut-off for low and high NIK and IKKα expression, whereas the lower quartile was used for RelB. The associations between protein expression and CSS are shown in Table 1. Expression of IKKα at any location was not associated with CSS. However, cytoplasmic NIK was significantly associated with poorer CSS (p=0.006, 13.3yrs v 11.3yrs, Figure 2A). 10 year CSS was stratified from 85% (low) to 68% (high, p=0.005). Similarly, cytoplasmic RelB was significantly associated with poorer CSS (p=0.041, 11.9yrs v 11.9yrs, Figure 2B). 10 year CSS was stratified from 88% (low) to 73% (high, p=0.030). Cytoplasmic NIK and RelB also significantly correlate, suggesting that they are both acting as part of the non-canonical pathway (p<0.001, Table 2). Trends towards poorer CSS were also seen for membrane NIK (p=0.057, 13.2yrs v 11.3yrs) and membrane IKKα (p=0.132, 12.9yrs v 11.6yrs). Again, membrane NIK strongly correlates with cytoplasmic NIK (P<0.001) and RelB (p<0.001, Table 2). Similarly, membrane IKKα shows associations with cytoplasmic RelB (p=0.084, Table 2). Interestingly, nuclear RelB showed a slight trend towards associations with poorer CSS (p=0.306, 12.8yrs v 12.2yrs) and strongly correlated with both cytoplasmic NIK and

RelB ($p<0.001$, Table 2). This suggests that pathway is active in RCC and NIK can modulate the pathway to translocate NIK to the nucleus to promote tumorigenesis.

The relationship between cytoplasmic NIK, RelB and clinicopathological characteristics were then examined (Table 2). Cytoplasmic NIK only associated with increased CSS ($p=0.014$). Whereas cytoplasmic RelB associated with increased grade ($p=0.020$), decreased KM grade ($p=0.019$) and trended towards associations with increased CSS ($p=0.079$). Cytoplasmic NIK and RelB were then entered into a multivariate cox regression survival analysis with stage, grade, and recurrence (Table 3). Stage (HR 1.63, $p=0.047$), recurrence (HR 9.75, $p<0.001$) and cytoplasmic NIK (HR 2.85, $p=0.009$) were independently associated with CSS.

DISCUSSION

This study shows that the non-canonical NF-kappa B pathway is associated with poor prognosis in RCC. Specifically, high cytoplasmic NIK and RelB are significantly associated with poor CSS, with membrane NIK/IKKα as well as nuclear RelB also showing similar trends. RelB, the most downstream member of the pathway, is associated with increased stage and decreased local inflammation, suggesting that the non-canonical pathway may downregulate local inflammatory infiltrate to facilitate tumor progression.

These results are similar to other studies that show NIK is a tumorigenic factor. In ovarian cancer cells, NIK has been shown to increase cell growth and tumorigenicity. In multiple myeloma, it has been proposed tumorigenesis depends upon NIK-driven p100/52 activation promoting B-cell survival.[11] NIK has also been shown to be a poor prognostic factor for glioblastoma.[6] Similarly, RelB has also been shown to be a poor prognostic factor in many solid tumors. In non-small cell lung cancer, RelB is associated with shorter overall survival, poorer differentiation, tumor invasion, lymph node metastasis, distant metastasis and TNM stage.[12] In ER+ve breast cancer, RelB and p52 were inversely correlated with ER levels and associated with poorer disease-free survival.[13] In bladder cancer, RelB and p52 levels have been shown to be increased in cancer tissue compared to normal bladder. Both RelB and p52 were also shown to be correlated with histological grade, stage and lymph node metastasis, suggesting that the non-canonical pathway promotes bladder cancer.[14]

Little is known about the role of the non-canonical pathway in renal disease including RCC. RelB has been shown to play a role in lymphocyte recruitment during acute

kidney injury when activated by TWEAK.[15] RelB has also been shown to play a role in renal ischemia-reperfusion injury (IRI). Silencing of RelB in a mouse model of IRI significantly attenuated IRI-induced renal dysfunction, with RelB silenced mice having a significant survival advantage.[16] However, literature on the effects of the non-canonical NFκB in RCC is lacking. One study shows that the non-canonical pathway is down-regulated in VHL-negative RCC cell lines and this results in diminished monocyte cell adhesion[17]. However, in this study we show that high expression of cytoplasmic NIK and RelB are associated with down regulation of the local inflammatory infiltrate and poor prognosis, with cytoplasmic NIK being an independent prognostic factor for these patients. Overall, this suggests that non-canonical pathway may be a potential therapeutic target for RCC.

To date the inflammatory effects of the NFκB pathway have mainly been attributed to the canonical p65/p50 subunit in conjunction with STAT3. However, NIK and RelB have previously been shown to be crucial for B-cell development,[18] suggesting that the non-canonical pathway also plays a role. The study also provides evidence that RelB can modulate the local inflammatory infiltrate in RCC patients.

CONCLUSION

In conclusion, the results of this study indicate that the non-canonical NFκB pathway is associated with poorer RCC patient prognosis through NIK and RelB. The study suggests that NIK regulates RelB activation, which in turn down regulates the local inflammatory infiltrate to facilitate tumor progression. Therefore NIK may be a useful

prognostic biomarker for this disease and both NIK and RelB may be potential therapeutic targets.

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Conflicts of Interest – The authors have no conflict of interest to disclose.

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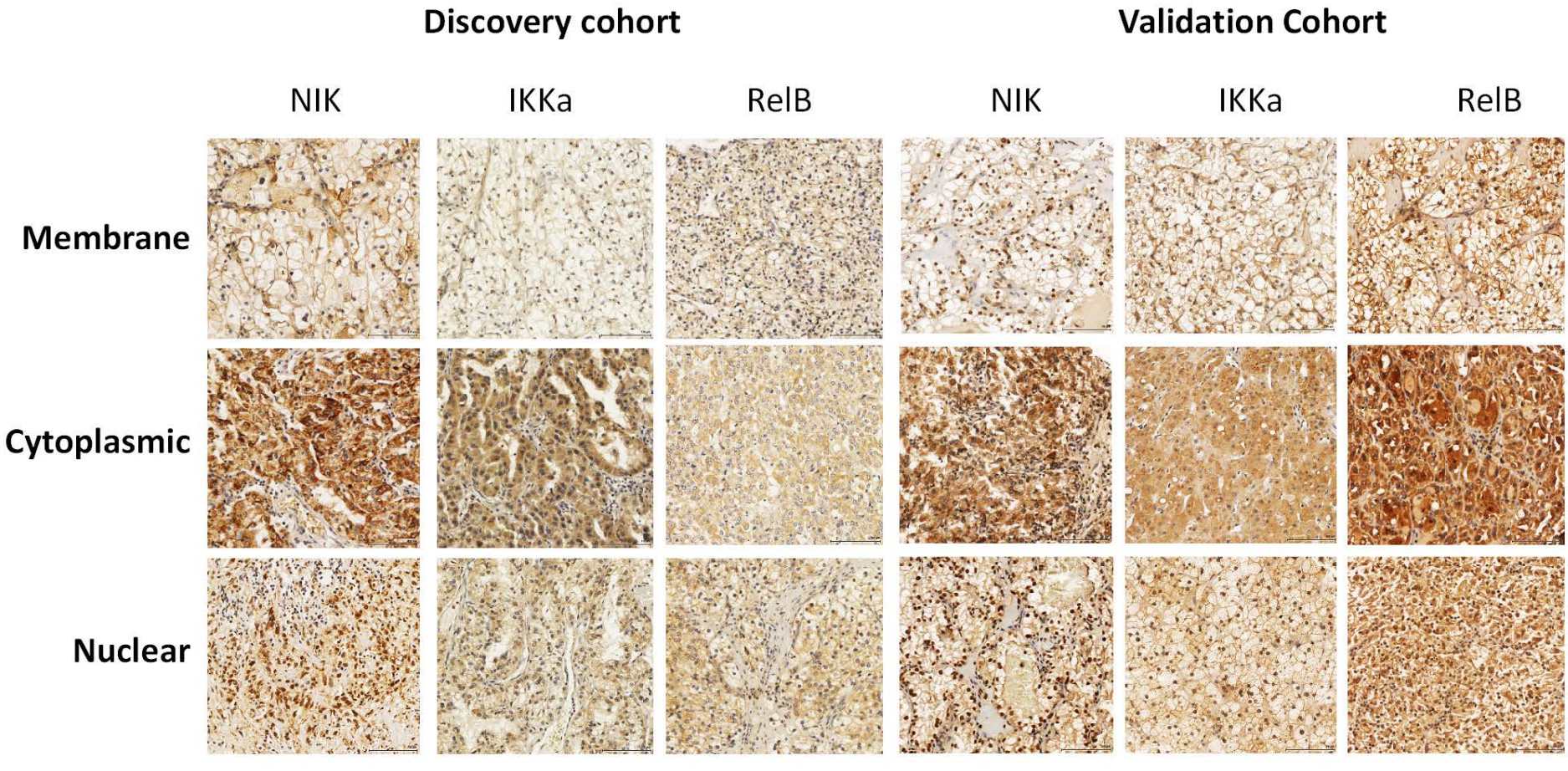
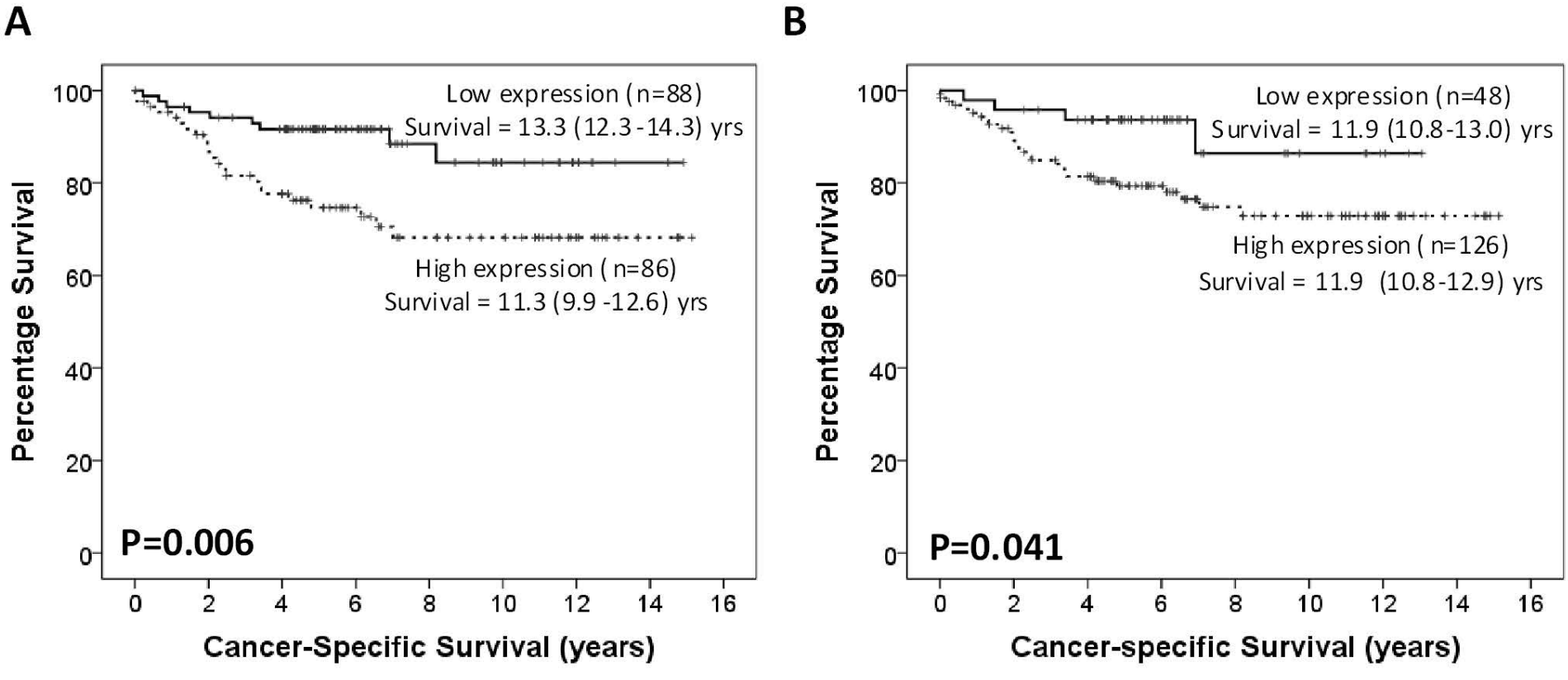
TITLES AND LEGENDS TO FIGURES

Figure 1. Immunohistochemical expression of NIK, IKKα and RelB in RCC patients. Representative images of high membrane, cytoplasmic and nuclear expression for NIK, IKKα and RelB. Images are taken from the patient with the highest expression of each protein at each location.

Figure 2. The non-canonical Nf-kappa B pathway associates with poorer cancer-specific survival. (A) Kaplan-Meier curve plotted for cytoplasmic NIK expression against cancer-specific survival. (B) Kaplan-Meier curve plotted cytoplasmic RelB expression against cancer-specific survival.


Discovery cohort
Validation Cohort
NIK
IKKa
RelB
NIK
IKKa
RelB
Membrane
Cytoplasmic
Nuclear


A
Low expression (n=88)
Survival = 13.3 (12.3-14.3) yrs
High expression (n=86)
Survival = 11.3 (9.9 -12.6) yrs
P=0.006
Percentage Survival
Cancer-Specific Survival (years)
B
Low expression (n=48)
Survival = 11.9 (10.8-13.0) yrs
High expression (n=126)
Survival = 11.9 (10.8-12.9) yrs
P=0.041
Percentage Survival
Cancer-specific Survival (years)

TABLES

Table 1. The relationship between the non-canonical NFκB pathway and cancer-specific survival in RCC patients (n=174)

		N (%)	10yr-CSS (SE)	P-value
NIK	**Membrane**	89 (51) 85 (49)	83 (5) 70 (6)	0.057
	Cytoplasmic	87 (50) 87 (50)	85 (5) 68 (6)	**0.006**
	Nuclear	87 (50) 87 (50)	75 (7) 77 (5)	0.535
IKKα	**Membrane**	89 (51) 85 (49)	80 (6) 73 (5)	0.132
	Cytoplasmic	87 (50) 87 (50)	77 (6) 75 (5)	0.502
	Nuclear	89 (51) 85 (49)	75 (7) 76 (5)	0.209
RelB	**Membrane**	42 (24) 132 (76)	79 (8) 76 (5)	0.821
	Cytoplasmic	48 (27) 126 (73)	88 (6) 73 (5)	**0.041**
	Nuclear	44 (25) 132 (75)	82 (7) 75 (5)	0.306

%=Percentage, CSS = cancer-specific survival, SE=standard error

Table 2. Correlations for members of the non-canonical NFκB in patients with RCC (n=174)

		NIK			IKKα			RelB		
		mem	cyto	nuc	mem	cyto	nuc	mem	cyto	nuc
NIK	mem		**<0.001**	**<0.001**	0.321	0.922	0.288	**<0.001**	**<0.001**	**0.003**
	cyto			0.075	0.711	0.161	0.778	**0.039**	**<0.001**	**<0.001**
	nuc				0.116	0.227	**0.043**	**0.010**	**0.025**	**<0.001**
IKKα	mem					**<0.001**	**<0.001**	**0.006**	0.086	0.084
	cyto						**<0.001**	0.154	0.120	0.722
	nuc							0.350	0.316	0.253
RelB	mem								**<0.001**	**<0.001**
	cyto									**<0.001**
	nuc									

Table 3. The relationship between clinicopathological characteristics and cytoplasmic NIK/RelB expression in RCC patients (n=174)

	Cytoplasmic NIK			Cytoplasmic RelB		
Patients	**Low (n=87)**	**High (n=87)**	**P value**	**Low (n=48)**	**High (n=126)**	**P value**
Age			0.104			0.664
≤60	37 (43)	47 (54)		22 (46)	61 (48)	
>60	50 (57)	40 (46)		26 (54)	65 (52)	
Stage			0.267			0.126
1	41 (47)	34 (39)		25 (52)	50 (40)	
2	15 (17)	15 (17)		8 (17)	22 (17)	
3	29 (34)	33 (38)		14 (29)	48 (38)	
4	2 (2)	5 (6)		1 (2)	6 (5)	
Grade			0.161			**0.020**
I	8 (9)	4 (5)		6 (13)	7 (6)	
II	27 (31)	27 (31)		11 (23)	44 (34)	
III	41 (47)	36 (42)		27 (56)	49 (39)	
IV	11 (13)	19 (22)		4 (8)	26 (21)	
Klintrup-Makinen grade			0.288			**0.019**
Strong	47 (54)	40 (46)		31 (65)	56 (44)	
Weak	40 (46)	47 (54)		17 (35)	70 (56)	
Recurrence			0.603			0.357
No	63 (72)	66 (76)		38 (79)	91 (72)	
Yes	24 (28)	21 (24)		10 (21)	35 (28)	
Survival			**0.014**			0.079
Alive	69 (80)	54 (62)		38 (79)	85 (67)	
Cancer-specific death	9 (10)	23 (26)		4 (8)	28 (23)	
Non-cancer death	9 (10)	10 (12)		6 (13)	13 (10)	

Table 4. The relationship between clinicopathological characteristics, the non-canonical NF-κB pathway and cancer-specific survival in RCC patients (n=174)

	Univariate analysis		Multivariate analysis	
Patients	**Hazard ratio (95% CI)**	***P*-value**	**Hazard ratio (95% CI)**	***P*-value**
Clinicopathological Characteristics				
Age (≤60/ >60 years)	1.00 (0.50-2.00)	0.991	-	-
Stage (1/2/3/4)	2.29 (1.53-3.44)	**<0.001**	1.63 (1.01-2.63)	**0.047**
Grade (I/II/III/IV)	2.15 (1.34-3.44)	**0.001**	1.28 (0.79-2.07)	0.321
Klitrup-Makinen Grade (Strong/weak)	0.88 (0.44-1.77)	0.725	-	-
Recurrance (No/Yes)	9.87 (4.43-22.00)	**<0.001**	9.75 (3.85-24.68)	**<0.001**
Non-canonical NF-kappa B pathway				
Cytoplasmic NIK (low/high)	2.80 (1.29-6.05)	**0.009**	2.85 (1.30-6.23)	**0.009**
Membrane NIK (low/high)	1.28 (0.64-2.58)	0.062	-	-
Cytoplasmic RelB (low/high)	2.85 (1.00-8.13)	**0.049**	1.24 (0.38-4.04)	0.727

CI= Confidence intervals